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Estimating novel potential drug targets of *Plasmodium falciparum* by analysing the metabolic network of knock-out strains *in silico*

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ABSTRACT

Malaria is one of the world's most common and serious diseases causing death of about 3 million people each year. Its most severe occurrence is caused by the protozoan *Plasmodium falciparum*. Biomedical research could enable treating the disease by effectively and specifically targeting essential enzymes of this parasite. However, the parasite has developed resistance to existing drugs making it indispensable to discover new drugs. We have established a simple computational tool which analyses the topology of the metabolic network of *P. falciparum* to identify essential enzymes as possible drug targets. We investigated the essentiality of a reaction in the metabolic network by deleting (knocking-out) such a reaction *in silico*. The algorithm selected neighbouring compounds of the investigated reaction that had to be produced by alternative biochemical pathways. Using breadth first searches, we tested qualitatively if these products could be generated by reactions that serve as potential deviations of the metabolic flux. With this we identified 70 essential reactions. Our results were compared with a comprehensive list of 38 targets of approved malaria drugs. When combining our approach with an *in silico* analysis performed recently [Yeh, I., Hanekamp, T., Tsoka, S., Karp, P.D., Altman, R.B., 2004. Computational analysis of Plasmodium falciparum metabolism: organizing genomic information to facilitate drug discovery. Genome Res. 14, 917–924] we could improve the precision of the prediction results. Finally we present a refined list of 22 new potential candidate targets for *P. falciparum*, half of which have reasonable evidence to be valid targets against micro-organisms and cancer.

1. Introduction

Malaria kills about 3 million people each year, of which more than 1 million are children under the age of five. In addition, up to half a billion people suffer from the effects of malaria (Snow et al., 2001). Although there are several different treatments for malaria, the parasite is becoming increasingly resistant to conventional antimalarial drugs. This has contributed to increasing morbidity and mortality. Four species of the *Plasmodium* genus cause human malaria. Among these, *Plasmodium falciparum* inflicts the most mortality and is responsible for about 90% of malaria deaths. Besides this, in biomedical research a considerable amount of data has been generated. Functional genomics of *P. falciparum* has been studied observed by the completion of sequencing the genome (Gardner et al., 2002), a variety of gene expression studies (e.g. Bozdech et al., 2003) and the setting up of a comprehensive metabolic reaction database (Yeh et al., 2004). Methodologically, such a reaction database can be used to construct and systematically analyse a metabolic network by linking pairs of reactions for which the product of one reaction is the substrate for the other. Such metabolic networks have been analysed with graph-based algorithms to identify drug targets in pathogenic organisms. For example, the term "damage" was defined to assess the enzymes that may serve as drug targets when their inhibition influences a broader amount of downstream reactions and products (Lemke et al., 2004; Mombach et al., 2006). Flux balance analysis (FBA) is a widely used and well-established method to assess the essentiality of genes for an organism (Becker et al., 2007; Edwards and Palsson,

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2000; Kauffman et al., 2003). FBA models the dynamics of integrated metabolic networks using dynamic mass balance equations. The concentration change of a metabolite over time is equal to the difference between the rates at which the metabolite is produced and consumed. In a steady state, the net concentration change of an inner metabolite in the network can then be modelled to be zero analogous to Kirchhoff's first law for electrical circuits (Edwards et al., 2002). However, FBA approaches need clear specifications defining nutrition availability and biomass production under specifically given environmental conditions (for a good overview of these aspects see (Schuetz et al., 2007)). Furthermore, concepts of choke-points and load-points were used to estimate if reactions are essential for the organism (Rahman and Schomburg, 2006; Yeh et al., 2004). In an extended graph theory model load-points were defined as nodes with a large number of *k*-shortest paths passing through them, which indicates high impact on metabolism. Load-points are defined as hot spots in the metabolic network (enzymes/metabolites) based on the ratio of the number of *k*-shortest paths passing through a metabolite/enzyme (in/out), and the number of nearest neighbour links (in/out) attached to it. This ratio is compared to the average load value in the network (Rahman and Schomburg, 2006). Choke-points uniquely consume or produce a certain metabolite, which may make them indispensable. It could be shown that inactivating choke-points leads to an organism's failure. For example, in *P. falciparum* d-aminolevulinate dehydratase (ALAD) has been considered as a choke-point (Yeh et al., 2004). In fact, d-aminolevulinate dehydratase is involved in heme biosynthesis and can serve as a valid antimalarial target (Bonday et al., 2000). With a choke-point analysis, Yeh et al. (2004) identified 216 enzymatic activities as catalysing choke-point reactions, assuming each enzyme has only one active site, unless annotated as multifunctional. If an enzyme catalysed at least one choke-point reaction, it was classified as a potential drug target. Within the 216 identified potential targets, they identified three targets of clinically proven drugs and 24 proposed drug targets with biological evidence (such as *in vitro* growth inhibition of the parasite with target inhibition). However, the precision (number of true predictions out of all predictions) of their approach is limited which makes it difficult for an experimentalist to choose the appropriate potential drug target when developing inhibitors as effective therapeutics.

In our work we used the data of the metabolic reaction database PlasmoCyc (Yeh et al., 2004) and developed an algorithm that analyses the topology of the metabolic network for *P. falciparum*. Basically, each reaction in the network was deleted (knocked out *in silico*), respectively. A breadth first search algorithm tested if the neighbouring compounds of the knocked out reaction could be produced by other reactions and pathways of reactions. In contrast to the choke-point approach, we checked the principle that deviations in the network could be used to replace the knocked out reaction. Fig. 1 illustrates this. The knocked out reaction is a choke-point, as metabolite D is uniquely produced by this reaction. However, D may not be essential for the organism if E and F can be produced using A and B as substrates for reactions r_4 and r_2, respectively (dashed lines in Fig. 1). To estimate the performance of our method, we defined a gold standard. For this, we assembled publically available data of approved and experimentally validated drugs for *P. falciparum* and yielded a list of 38 reactions within our network that are successfully targetable. Note that, this is not a true gold standard in the sense that a lot of true positves may have not been discovered yet. We used the gold standard for estimating the prediction performance of our algorithm, in comparison to the choke-point approach predicting and optimising an enrichment of discovered drug targets (enrichment of reactions from the gold standard). In addition, one can assume that enriching discovered drug targets comprises an enrichment of non-discovered drug targets, which then can be found in the list of false positive predictions.

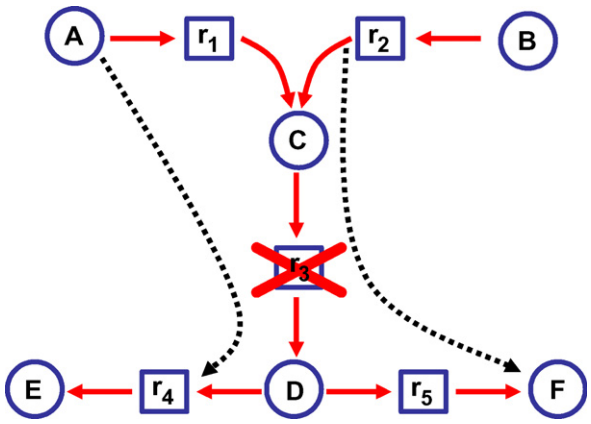

Fig. 1. The knocked out reaction is a choke-point but may not be essential for the organism if the dashed lines exist in the metabolic network. Our approach inspects the network for such deviations (see text). Reactions are illustrated as boxes, metabolites as circles.

In comparison to the choke-point analysis (Yeh et al., 2004), we were able to improve the prediction results when combining our method with the choke-point method. Using the choke-point analysis alone yielded an accuracy of 68% and a precision of 19%, whereas, applying the choke-point analysis together with our method, yielded an increased accuracy of 88% and a precision of 29%. Finally, we analysed the "false" positives. This list may serve as candidates for new drug targets. We tested the sequences of these candidates for sequence homology to the human genome to exclude severe physiological side effects when targeting. Finally, an assembled list of 22 potential new drug targets is presented. Eleven of which are reported in the literature as drug targets against micro-organisms and cancer.

2. Materials and methods

2.1. Reconstructing the metabolic network

All metabolic reactions were extracted from the PlasmoCyc database ((Karp et al., 2005), http://www.biocyc.org, Version 10.5). As described elsewhere (König and Eils, 2004) a connected graph was established by defining neighbours of reactions: two reactions were neighbours if a metabolite existed that was the product of one reaction and the substrate for the other. This yielded a bipartite graph of alternating reaction and metabolic compound nodes. Metabolites that were highly connected and therefore pathway unspecific, such as water, oxygen and ATP were discarded. This yielded a network with 554 metabolites and 575 reactions. As there was no information available, each reaction was considered to be reversible.

2.2. The algorithm

Basically, the producibility of a network having a deleted reaction ("mutated network" in the following) was compared to the wild-type network without the deletion. These comparisons were performed for each reaction. A reaction was given out as essential if producing products downstream of it was indicated to hamper. In detail, the procedure was as follows:

(1) A reaction from the network was selected.
(2) A set of products S was assembled containing all metabolites that were produced downstream from the deleted reaction. To gain a higher variability, we added also all metabolites of the products of all reactions that had theses metabolites as substrates.
(3) From this set, we sampled 1000 smaller subsets s_i, $i = 1, \ldots, 1000$, containing randomly drawn metabolites from S. s_i had got a size of 30% of S.
(4) i was set to 1.
(5) The mutated network was tested if it was able to produce all elements of s_i using all reactions and metabolites of the network. To avoid trivial solutions, metabolites that were contained in s_i could not serve as substrates. If producing the subset s_i was possible, p_m was set to one, otherwise to zero. Furthermore, the mutated network was tested for its effectiveness. For this, a minimum number of necessary reactions and metabolites were assessed by a greedy approach: a list of all reactions (except the knocked out reaction) was composed by sampling without replacement. Starting from the top (and ending at the bottom) of the list, each reaction was tested if it could be discarded without losing the possibility to produce the elements of s_i. This test was performed by a common breadth first search on graphs (see, e.g. Cormen et al., 1995). Every time this test failed, the respective reaction was placed back into the network and the next one in the list was tested. The resulting number of reactions was r_m. The same was performed with a list of all metabolites of the network yielding an estimated minimal set of necessary metabolites m_m.
(6) The same tests (using the same lists of reactions and metabolites) were performed with the wild-type network yielding p_w (equaling one if producing all products of s_i, otherwise zero) and the assessed minimal number of reactions r_w and metabolites m_w.
(7) Steps (5) and (6) were repeated for every $i = 1$ to 1000. Mean values for the variables p_m, p_w, m_m, m_w, r_m, r_w were calculated yielding $\langle p_m \rangle$, $\langle p_w \rangle$, $\langle m_m \rangle$, $\langle m_w \rangle$, $\langle r_m \rangle$, $\langle r_w \rangle$. A reaction was estimated to be essential if

$$\langle p_m \rangle < \langle p_w \rangle \text{ and } (\langle r_m \rangle > \langle r_w \rangle \text{ or } \langle m_m \rangle > \langle m_w \rangle).$$

Additionally, reactions with connectivity higher than 10 were discarded to restrict the predictions to non-hub like reactions.
(8) Steps (2)–(7) were performed for every reaction in the network.

2.3. The gold standard and statistical settings

We assembled a list of proposed drug targets from the literature. We used the list of Yeh et al. (2004) comprising of three targets of clinically proven drugs and 24 proposed drug targets with biological evidence, such as *in vitro* growth inhibition of *P. falciparum* (for details, see Yeh et al., 2004). Additionally, we found further drug targets when scanning a variety of established databases, i.e. Drugbank (www.redpoll.pharmacy.ualberta.ca/drugbank), TDR Target Database (www.tdrtarget.org) and the database for Malaria Parasite Metabolic Pathways by Hagai Ginsburg (http://www.sites.huji.ac.il/malaria/, see also (Ginsburg, 2006)). To equally compare all predictions with the gold standard, every reaction of the network was mapped to its corresponding enzyme classification (EC) number. For performance estimations, reactions without an EC number were not taken into account. With this, our network contained 38 reactions from the gold standard and consisted of a total of 411 reactions. The complete list of the gold standard is given in Table 1. To yield a valid comparison of the algorithms we did not take the reactions into account that were not in the network. As our network was constructed as a connected graph, we discarded all reactions that were not joined with the bulk of the graph. With this construction the following reactions which have been shown to serve as valid drug targets where not included in the network: enoyl-[acyl-carrier protein] reductase (NADH) (EC 1.3.1.9) (Surolia and Surolia, 2001), DNA-directed DNA polymerase (EC 2.7.7.7) (Barker et al., 1996), thioredoxin reductase (NADPH) (EC 1.8.1.9) (Krnajski et al., 2002), lysophospholipase (EC 3.1.1.5) (Zidovetzki et al., 1994) and DNA-directed RNA polymerase (EC 2.7.7.6) (Lin et al., 2002).

3. Results

Three different scenarios were compared, (i) the choke-point analysis as described recently (the choke-point list was taken from Yeh et al., 2004, for details see there), (ii) our method alone, and (iii) a combination of both where a reaction was assessed as essential if it was a choke-point *and* essential according to our method. We evaluated the performance of all three approaches with the gold standard consisting of reactions that are targets of approved and tested drugs. The results are given in Tables 2–4, respectively. Note that the tables represent confusion matrices showing true positives, false positives, false negatives and true negatives, from left to right and top to bottom, respectively. Using the choke-point approach alone yielded an accuracy of 68% (accuracy = all correct predictions divided by all predictions $\times$ 100%). With our approach alone, we yielded an accuracy of 80%. The best accuracy of 88% was achieved when taking the intersection of essential reactions from the choke-point analysis and our method. We were especially interested in the precision (true positives of all positive predictions) to serve experimentalists with a reduced number of wrong estimates and therefore giving them a reduced load of non-profitable assignments. Also here the results were best when combining both approaches (combined: 29%, choke-point alone: 19%, our method alone: 17%). Note that the combined approach yielded a rather low sensitivity (23%). A high sensitivity could be reached by assembling the *union* of both algorithms (sensitivity = 82%, accuracy = 60%, precision = 16%). The complete list for the union is given in the supplement (supplementary Table S1). The parameter s_p for step 3 in the algorithm was set to its value of $s_p = 30\%$ after initial trials with $s_p = 20\%$, 30% and 40%. With $s_p = 20\%$ we got lower accuracy and precision by 1% and 4%, respectively. Choosing $s_p = 40\%$ yielded slightly better values (accuracy: +1%, precision: +2%) compared to $s_p = 30\%$. However, with $s_p = 40\%$ we could not get the products ($p_w = 0$) even for the wild-type for subsets s_i in more than 990 out of 1000 runs for 8% of all tested reactions (compared to 0.3% for $s_p = 30\%$ and $s_p = 20\%$), making the comparison between wild-type and mutant for these reactions less reliable. This behaviour is due to a blocking effect as each selected substrate of s_i cannot be used as an intermediate to obtain the products from the other substrates. The list of the gold standard may not be complete and rather lack of putative new drug targets. Therefore, we took all enzymes of the positive predictions from our combined analysis as putative novel drug targets. From these enzymes, all corresponding genes were compared to all transcripts of the human genome using BLAST (Altschul et al., 1997) and the ENSEMBL database (Hubbard et al., 2007). Arginine-tRNA ligase showed some homology with *E*-value 4×10^{-4} and may need more detailed homology investigations of its active domain. For the rest, we did not find any significant homologies (all *E*-values > 0.01). Table 5 shows the resulting list with the best hits and their respective *E*-values.

3.1. Comparing our predicted drug targets with the literature

We searched through the literature in-depth to compare our results (Table 5) with treatments and findings for other micro-

Table 1
The gold standard.

EC	Reaction	Found by our method	Choke-points	Found by a combination	Reference
1.1.1.205	IMP dehydrogenase	No	No	No	Webster and Whaun (1982)
1.17.4.1	Ribonucleoside-diphosphate reductase	No	Yes	No	Chakrabarti et al. (1993)
1.2.4.4	3-Methyl-2-oxobutanoate dehydrogenase (lipoamide)	Yes	Yes	Yes	Lau et al. (1990)
1.3.3.1	Dihydroorotate oxidase	No	No	No	Baldwin et al. (2005), Boa et al. (2005), Heikkila et al. (2006), McRobert and McConkey (2002)
1.3.99.1	Succinate dehydrogenase	No	No	No	Suraveratum et al. (2000)
1.5.1.3	Dihydrofolate reductase	No	Yes	No	Le Bras and Durand (2003)
1.6.5.3	NADH dehydrogenase (ubiquinone)	No	Yes	No	Krungkrai et al. (2002)
2.1.1.100	Protein-*S*-isoprenylcysteine-*O*-methyltransferase	No	No	No	Baron et al. (2007), Winter-Vann et al. (2005)
2.1.1.45	Thymidylate synthase	No	Yes	No	Jiang et al. (2000)
2.1.1.64	3-Demethylubiquinone-9,3-*O*-methyltransferase	No	Yes	No	Massimine et al. (2006)
2.3.1.15	Glycerol-3-phosphate-*O*-acyltransferase	No	Yes	No	Ginsburg (2006)
2.3.1.41	3-Oxoacyl-[acyl-carrier protein] synthase	No	Yes	No	Waller et al. (2003)
2.4.2.1	Purine-nucleoside phosphorylase	No	Yes	No	Kicska et al. (2002)
2.4.2.8	Hypoxanthine phosphoribosyltransferase	No	No	No	Sarma et al. (1998)
2.5.1.15	Dihydropteroate synthase	No	Yes	No	Triglia et al. (1997)
2.5.1.16	Spermidine synthase	Yes	Yes	Yes	Haider et al. (2005)
2.5.1.18	Glutathione transferase	Yes	No	No	Fritz-Wolf et al. (2003), Harwaldt et al. (2002), Liebau et al. (2002), Perbandt et al. (2004)
2.5.1.19	3-Phosphoshikimate 1-carboxyvinyltransferase	Yes	Yes	Yes	Roberts et al. (1998)
2.5.1.21	Farnesyl-diphosphate farnesyltransferase	Yes	Yes	Yes	Chakrabarti et al. (2002)
2.7.1.32	Choline kinase	Yes	No	No	Ancelin and Vial (1986)
3.1.3.56	Inositol-1,4,5-trisphosphate 5-phosphatase	Yes	Yes	Yes	Ogwan'g et al. (1993)
3.1.4.12	Sphingomyelin phosphodiesterase	No	Yes	No	Hanada et al. (2002)
3.1.4.17	3′,5′-Cyclic-nucleotide phosphodiesterase	Yes	Yes	Yes	Yuasa et al. (2005)
3.3.1.1	*S*-adenosyl-L-homocysteine hydrolase	No	Yes	No	Kitade et al. (1999), Shuto et al. (2002)
3.5.4.4	Adenosine deaminase	No	No	No	Tyler et al. (2007)
3.6.1.17	Bis(5′-nucleosyl)-tetraphosphatase (asymmetrical)	No	Yes	No	Guranowski et al. (2003)
4.1.1.17	Ornithine decarboxylase	No	Yes	No	Berger (2000)
4.1.1.23	Orotidine-5′-phosphate decarboxylase	Yes	No	No	Krungkrai et al. (2005), Scott et al. (1986), Seymour et al. (1994)
4.1.1.50	Adenosylmethionine decarboxylase	No	Yes	No	Wright et al. (1991)
4.1.2.13	Fructose-bisphosphate aldolase	No	Yes	No	Wanidworanun et al. (1999)
4.2.1.24	Delta-aminolevulinic acid dehydratase	No	Yes	No	Bonday et al. (2000)
4.2.3.5	Chorismate synthase	No	Yes	No	McRobert and McConkey (2002)
4.4.1.5	Lactoylglutathione lyase	Yes	Yes	Yes	Thornalley et al. (1994)
6.1.1.3	Threonine-tRNA ligase	Yes	Yes	Yes	Ruan et al. (2005)
6.1.1.7	Alanine-tRNA ligase	No	Yes	No	Corvaisier et al. (2003)
6.3.2.2	Gamma-glutamylcysteine synthetase	Yes	Yes	Yes	Meierjohann et al. (2002)
6.3.5.5	Carbamoyl-phosphate synthase (glutamine-hydrolysing)	No	Yes	No	Flores et al. (1997)
6.4.1.2	Acetyl-CoA carboxylase	No	No	No	Waller et al. (2003)

organisms and cancer. For one of our predicted drug targets we found that it has already been described to be a drug target in *P. falciparum* (marked by ****, Table 5 and see below), for four we found clear evidence to be targeted against other microbial diseases (***), for two we found anticancer drugs (**) and for four we found reasonable evidence to be targeted against other diseases (*). Furthermore, we compiled some interesting information for four enzymes which may not serve as drug targets (no asterisks). In the following, our findings are described in more detail.

***EC 2.1.2.9: Inhibiting methionyl-tRNA formyltransferase caused impaired growth in *Escherichia coli* (Mazel et al., 1994). EC 2.4.2.11: Nicotinate phosphoribosyltransferase reduces oxidative stress (Hara et al., 2007). Zerez et al. (1990) observed a threefold increase of Nicotinate phosphoribosyltransferase

Table 2
Results using the choke point approach alone (accuracy: 68%, precision: 19%).

	Truth (state of the art)		
	Known drug target ("essential")	Not known as a drug target ("non-essential")	Sum
Prediction			
Essential	28	123	151
Non-essential	10	250	260
Sum	38	373	411

Table 3
Results using our method alone (accuracy: 80%, precision: 17%).

	Truth (state of the art)		
	Known drug target ("essential")	Not known as a drug target ("non-essential")	Sum
Prediction			
Essential	12	58	70
Non-essential	26	315	341
Sum	38	373	411

Table 4
Results assessing a reaction essential if it is a choke point *and* essential according to our method (accuracy: 88%, precision: 29%).

	Truth (state of the art)		
	Known drug target ("essential")	Not known as a drug target ("non-essential")	Sum
Prediction			
Essential	9	22	31
Non-essential	29	351	380
Sum	38	373	411

in infected red blood cells. However, this may be difficult to target specifically as the human red blood cell produces this enzyme.

**EC 2.4.2.30: NAD(+) ADP-ribosyltransferase is a well known anticancer drug (Ratnam and Low, 2007).

****EC 2.5.1.46: Deoxyhypusine synthase is a known drug target for *P. falciparum* (Moritz et al., 2004).

*EC 2.7.1.1: Hexokinase activity in cell-free *Plasmodium berghei* was observed to be 35 and 5 times higher as compared to normal and *P. berghei*-infected mouse erythrocytes, respectively (Kumar and Banyal, 1997). Therefore, blocking of hexokinase may yield synergistic effects when combining it with blocking hemoglobin ingestion and degradation as the major nutrient uptake channel.

*EC 2.7.1.35: Besides vitamin B6 (Ebadi et al., 1982), aminphylline also is blocking the enzyme pyridoxal kinase (Delport et al., 1990). In turn, aminophylline is aiding antibiotics to prevent resistance in *S. aureus* and *P. aeruginosa* (Hosseinzadeh et al., 2006).

Enzymes EC 2.7.1.50 and EC 2.7.4.7 are involved in the biosynthesis of the vitamin thiamine. Blocking these enzymes may stop the biosynthesis of thiamine. However, thiamine may not be essential for the parasite in rich media.

*EC 2.7.4.9 (thymidylate kinase/DTMP kinase): We could not find a drug targeting thymidylate kinase. Thymidylate kinase is one step in the biosynthesis pathway of deoxythymidine triphosphate (dTTP). dTTP is needed for, e.g. synthesising DNA. Nevertheless, is has been shown very recently that the direct upstream reaction, thymidine kinase, has been successfully targeted for inhibiting growth of *Mycobacterium tuberculosis* (Van Daele et al., 2007). We therefore suggest thymidylate kinase as a good potential drug target with similar effects.

***EC 2.7.8.11: It has been shown that phosphatidylinositol synthase is essential in *Trypanosoma brucei* (Martin and Smith, 2006).

***EC 3.1.2.6: Hydroxyacyl glutathione hydrolase has been suggested as an antimicrobial drug against *Leishmania* (Padmanabhan et al., 2006).

EC 3.5.1.19: Nicotinamidase is used in *M. tuberculosis* to metabolite pyrazinamide into pyrazionic acid which in turn gives the antibacterial effect of the treatment. Hence, in this case treating a patient by blocking nicotinamidase yields an antagonistic response when administering pyrazinamide at the same time (Scorpio and Zhang, 1996). However, we could not find any literature describing the effects when exclusively inhibiting nicotinamidase.

***EC 4.2.1.60: 3-Hydroxydecanoyl-ACP dehydrase is essential in *M. tuberculosis* and serves as a good candidate for drug targets (Sacco et al., 2007).

**EC 6.1.1.19: Arginine-tRNA ligase serves as a potential drug target against cancer of the pancreas (Bence and Crooks, 2003).

*EC 6.2.1.3: It is assumed that long-chain-fatty-acid CoA ligase is inhibited by a rice herbicide preventing the biosynthesis of very-long-chain fatty acids (Takahashi et al., 2001). Note that this reaction may be used in *Plasmodium* within the fatty acid beta-oxidation II pathway which is normally found in plants.

4. Discussion

We have established a simple method that analyses metabolic pathways and identifies essential reactions as potential drug targets in the metabolic network of *P. falciparum*. Using the target reactions of approved drugs as a gold standard, we yielded more precise predictions when combining our method with the established approach of choke-point analysis. This makes sense, as additional to the choke-point analysis we carefully checked for alternative pathways that may generate the products of the tested knock-out reactions. In turn, our method alone also yielded a lower precision and accuracy compared to the combined approach. This may be due to the fact that our algorithm simply searches for any

Table 5
Predicted potential drug targets.

EC	Reaction	Evidence	Genes	Human homologs	*E*-value
2.1.2.9	Methionyl-tRNA formyltransferase	***	MAL13P1.67	ENST00000373665	0.19
2.4.1.119	Dolichyl-diphosphooligosaccharide–protein glycosyltransferase		PFI0960W	ENST00000306726	0.034
2.4.2.11	Nicotinate phosphoribosyltransferase		MAL6P1.137	ENST00000370856	0.19
2.4.2.30	NAD(+) ADP-ribosyltransferase	**	PFI1005W	ENST00000282892	0.052
2.5.1.	Glutamyl-tRNA(Gln) amidotransferase		MAL6P1.78	ENST00000340159	0.009
2.5.1.46	Deoxyhypusine synthase	****	PF14_0125	ENST00000352853	0.54
2.7.1.1	Hexokinase	*	MAL6P1.189	ENST00000240487	0.14
2.7.1.35	Pyridoxal kinase	*	MAL6P1.266	ENST00000234179	0.011
2.7.1.50	Hydroxyethylthiazole kinase		PFL1920C	ENST00000346134	0.005
2.7.4.7	Phosphomethylpyrimidine kinase		PFE1030C	ENST00000382103	0.086
2.7.4.9	Thymidylate kinase	*	PFL2465C	ENST00000340245	0.90
2.7.7.2	FMN adenylyltransferase		PF10_0147	ENST00000256103	0.091
2.7.8.-	Cardiolipin synthetase		MAL6P1.97	ENST00000233710	0.66
2.7.8.11	CDP-diacylglycerol–inositol 3-phosphatidyltransferase	***	MAL13P1.82	ENST00000321998	0.19
3.1.2.6	Hydroxyacylglutathione hydrolase	***	PFL0285W	ENST00000389580	0.35
3.5.1.19	Nicotinamidase		PFC0910W	ENST00000294671	0.12
4.1.2.4	Deoxyribose-phosphate aldolase		PF10_0210	ENST00000356689	0.29
4.2.1.17	Enoyl-CoA hydratase		PF10_0167	ENST00000335407	0.16
4.2.1.60	3-Hydroxydecanoyl-[acyl-carrier protein] dehydratase	***	PF13_0128	ENST00000297933	0.25
4.2.1.70	Pseudouridylate synthase		PFB0890C	ENST00000370920	0.37
6.1.1.19	Arginine-tRNA ligase	**	PFL0900C	ENST00000231572	2e-04
6.2.1.3	Long-chain-fatty-acid-CoA ligase	*	PF14_0761	ENST00000038037	0.23
			PFB0695C	ENST00000373480	0.063

kind of deviations in the network that biochemically may not always serve as a valid replacement. This is especially the case, when analysing higher connected reactions. After testing a variety of different upper boundary values, we restricted the connectivity of predicted essential reactions to an optimal upper limit of 10. However, drugable reactions with higher connectivity could therefore not be discovered: The mean connectivity of our true positive predictions was 2.6. In contrast, essential reactions that we could not discover (false negatives) had an increased mean connectivity of 6.5. For the choke-point approach both values were comparable (mean connectivity was 5.1 for true positives and 5.4 for false negatives). This emphasizes to apply the combination of both approaches: assembling the *union* of the predictions from both methods yielded a good sensitivity (82%) whereas their intersection yielded an improved precision (29%) and a good accuracy of 88%. However, the precision of 29% was also not very high. The reason for this is twofold: we need to improve our algorithm *and* some of our false positive predictions are indeed valid new drug targets. With at least a reasonable evidence, this could be experimentally shown for half of these targets (11 out of 22, see Section 3). In addition, if regarding the KEIO knock-out collection for *E. coli*, in which every open reading frame was knocked out and tested for its essentiality, around 300 ORFs were observed to be essential (Baba et al., 2006). We mapped these ORFs to their according reactions and found around 100 metabolic reactions to be essential in *E. coli*. Transferring this order of magnitude to our case, we estimate that until now only about half of all essential reactions in *P. falciparum* may have been experimentally validated and targeted so far. In comparison to the choke-point analysis, we yielded a rather low sensitivity (our method: 32%, choke-point: 74%, combined: 24%). However, it was not our aim to predict a large list of potential targets with a high number of false positives. We calculated a smaller defined list of concrete candidate targets to be tested in the lab. For our concept, no initial settings are needed. This makes its application easier in comparison to flux balance analyses, where the environmental conditions need to be defined, such as availability of nutrients, the carbon sources and temperature. In contrast, we restricted our method to scan over the local topological properties of the network, around the investigated reaction. Such a concept may be combined with a flux balance analysis by defining the investigated region of the network and restricting to local subnets, making FBA more independent from environmental settings. Furthermore, concepts like estimating the damage (Lemke et al., 2004) may be further included in such approaches by taking the substantially effected downstream regions of the metabolism into account. Knocking-out open reading frames has been performed in high throughput for several organisms including *E. coli* (Baba et al., 2006), yeast (Giaever et al., 2002) and *Candida albicans* (Roemer et al., 2003). Even though such studies are costly and labour intensive it is quite likely that they will also be performed with *Plasmodium* in the near future. It will be interesting to compare our *in silico* predictions with such experimental data. But note that in contrast to these experimental approaches, our *in silico* predictions are feasible to also handle multiple knock-outs on a high throughput yielding possible effective drug combinations. We plan to do this when some data of at least a limited amount of drug combinations has been made available. In conclusion, our approach is computational inexpensive and simple to implement, it limits time and cost consuming wet lab experiments and has got the potential to serve as a valid technique to be combined with other established graph-based investigations of the metabolism. In principle, such *in silico* investigations can be performed for any organism if its genome and its inferred metabolic network is discovered sufficiently enough.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.meegid.2008.01.007.

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